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Thermal Generation of 3-Amino-4,5-dimethylfuran-2(5*H*)-one, the Postulated Precursor of Sotolone, from Amino Acid Model Systems Containing Glyoxylic and Pyruvic Acids

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ABSTRACT: 4,5-Dimethyl-3-hydroxy-2(5*H*)-furanone (sotolone), a naturally occurring flavor impact compound, can be isolated from various sources, especially fenugreek seeds. It can also be thermally produced from intermediates generated from the Maillard reaction such as pyruvic and ketoglutaric acids, glyoxal, and 2,3-butanedione. A naturally occurring precursor of sotolone, 3-amino-4,5-dimethyl-2(5*H*)-furanone, was thermally generated for the first time from pyruvic acid and glycine or from glyoxylic acid and alanine model systems. Isotope labeling studies have implicated 4,5-dimethylfuran-2,3-dione as an intermediate that can be converted into 3-amino-4,5-dimethyl-2(5*H*)-furanone through Strecker-like interaction with any amino acid. Furthermore, these studies have also indicated the presence of two pathways for the formation of 4,5-dimethylfuran-2,3-dione, one requiring pyruvic acid and a formaldehyde source and the other requiring glyoxylic acid and acetaldehyde. Self-aldol condensation of pyruvic acid followed by lactonization and further aldol reaction with formaldehyde can generate the same intermediate as the self-aldol addition product of acetaldehyde with glyoxylic acid followed by lactonization. The pyruvic acid pathway was found to be a more efficient route than the glyoxylic acid pathway. Furthermore, the pyruvic acid/glycine model system was able to generate sotolone in the presence of moisture, and in the presence of ammonia, commercial sotolone was converted back into 3-amino-4,5-dimethyl-2(5*H*)-furanone.

KEYWORDS: glyoxylic acid, pyruvic acid, glycine, sotolone, isotope labeling, 3-amino-4,5-dimethylfuran-2(5*H*)-one, Strecker reaction

INTRODUCTION

Chemical reactions occurring in food during thermal processing generating structures identical to those formed enzymatically such as during metabolic processes occurring in plants are rarely encountered. Furaneol, Strecker aldehydes, and some pyrazines[1] are a few examples of important Maillard reaction products that at the same time are also known to be formed enzymatically in various fruits and vegetables. Similarly, sotolone, 4,5-dimethyl-3-hydroxy-2(5*H*)-furanone, was isolated from various natural sources such as fenugreek seeds,[2] mushrooms,[3] and lovage[4] as well as from processed foods such as cane sugar,[5] botrytized wines,[6] aged sake,[7] aged Port wine,[8] and coffee.[9] Sotolone is an important flavor impact compound with a very low threshold value of 0.02 ng/L air.[9] Its aroma characteristics change from caramel-like at low concentrations to curry-like at high concentrations.[10] Thermally, it can be formed through intermediates generated from the Maillard reaction such as pyruvic and ketoglutaric acid, the latter originating from glutamic acid.[10,11] In aged sake, sotolone can be produced by condensation of ketobutyric acid and acetaldehyde, both being acid decomposition products of threonine.[7] Sotolone can also be detected in aqueous solutions of glyoxal/2,3-butanedione at pH 5.[12] Sotolone, the character-impact compound of fenugreek seed (*Trigonella foenum-graecum* L.), was first reported by Rijkens and Boelens;[13] however, its metabolic origin remains unclear. There are indications that oxidative deamination of 4-hydroxyisoleucine (HIL), the most abundant amino acid in fenugreek seed, or its corresponding lactone, 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone,[14] could be possible precursors of sotolone in fenugreek[15] (see Figure 1). In addition, 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) was reported by Rapior et al.[3] to be also present in the cultures of *Lactarius helvus*, a mushroom from Europe producing sotolone, and in fenugreek seed (*T. foenum-graecum*) by Peraza-Luna et al.[15] Although both amino compounds have been postulated as precursors of sotolone, their possible role in the metabolic pathways of sotolone formation has not been investigated. 3-Amino-4,5-dimethyl-2(5*H*)-furanone (**1**) can be hydrolyzed into sotolone as shown in Figure 1. Here we report for the first time the mechanism of thermal generation of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) from amino acid model systems containing glyoxylic and pyruvic acids. Such precursors could be used as controlled delivery systems of sotolone aroma in processed foods.

MATERIALS AND METHODS

Reagents and Chemicals. Glycine (99%), L-alanine (99%), pyruvic acid (98%), glyoxylic acid (98%), glycine hydrochloride (98%), pyruvic acid sodium salt (98%), sotolone (3% solution), ammonium chloride, and paraformaldehyde (95%) were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada). The labeled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), [^{15}N]glycine (98%), [^{13}C-1]pyruvic acid sodium salt (99%), [^{13}C-2]pyruvic acid sodium

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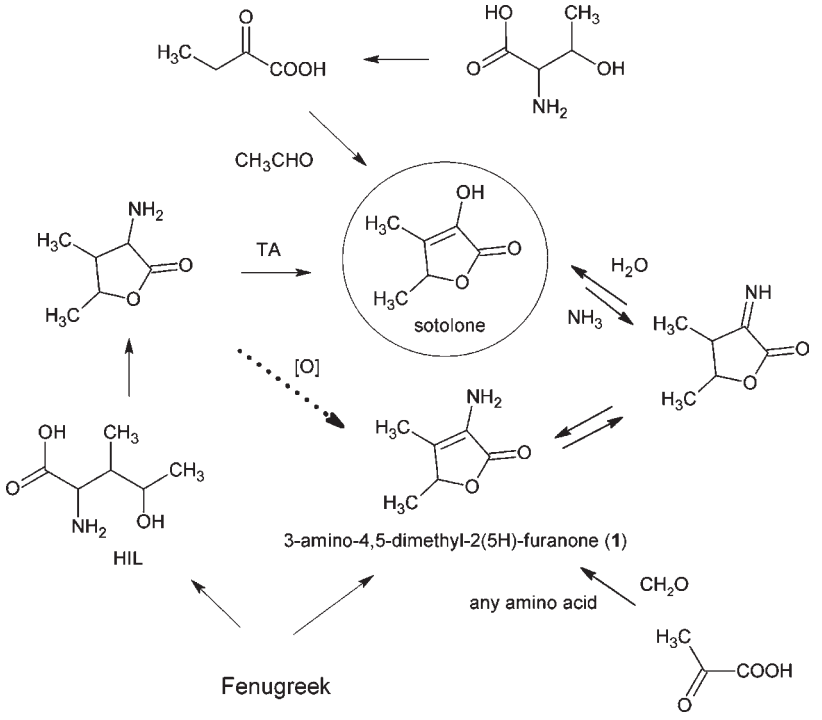

Figure 1. Known precursors of sotolone. Dotted arrows indicate proposed pathways. TA, transaminase; HIL, 4-hydroxyisoleucine.

salt (99%), [^{13}C-3]pyruvic acid sodium salt (99%), [^{13}C-1]alanine (99%), [^{13}C-2]alanine (99%), and [^{15}N]alanine (98%) were purchased from Cambridge Isotope Laboratories (Andover, MI). Fenugreek seeds were purchased from a local market.

Pyrolysis−Gas Chromatography−Mass Spectrometry (Py-GC-MS). The Py-GC-MS analysis was performed according to a procedure described by Guerra and Yaylayan[16] with some modifications. A Varian CP-3800 GC equipped with a sample preconcentration trap (SPT) filled with Tenax GR was coupled to a Varian Saturn 2000 mass spectrometry detector (Varian, Walnut Creek, CA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA). The samples were analyzed on a DB-5MS column (5% diphenyl, 95% dimethylpolysiloxane) with column dimensions of 50 m length × 0.2 mm internal diameter × 33 μm film thickness (J&W Scientific, ON, Canada) using helium as the carrier gas. Two milligrams of sample mixtures containing 3:1 ratio of amino acid to keto acid, 3:1:0.5 ratio of glycine to glyoxylic acid to pyruvic acid, and 1:1 or 3:1 ratio of paraformaldehyde to pyruvic acid or 3 mg of ground fenugreek seed was packed inside a quartz tube (0.3 mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 200 °C for 20 s under helium atmosphere. For reactions performed under moisture, water (5 μL) was added to silica gel (1 mg) that was physically separated from the reaction mixture by a layer of glass wool and located at the exit end of the quartz tube. The volatiles after pyrolysis were concentrated on the sample preconcentration trap (SPT), trapped at 50 °C, and subsequently directed toward the GC column for separation. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a delayed (30 s) pressure pulse of 70 PSI for the first 4 min and maintained with a constant flow of 1.5 mL/min for the rest of the run. The GC oven temperature was set at −5 °C for the first 5 min using CO_2 as the cryogenic cooling source and then increased to 50 °C at a rate of 50 °C/min. Then, the oven temperature was again increased to 270 °C at a rate of 8 °C/min and kept at 270 °C for 5 min. The MS transfer-line temperature was set at 250 °C, the manifold temperature was set at 50 °C, and the ion trap temperature was set at 175 °C. The ionization voltage of 70 eV was used, and EMV was set at 1500 V. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

Table 1. Percent Incorporation of Labeled Atoms from Glycine and Pyruvic Acid in *m/z* 127 of 3-Amino-4,5-dimethyl-2(5*H*)-furanone (1)

labeled reactant	M	M + 1	M + 2
[^{13}C-1]glycine[a]	0	0	0
[^{13}C-2]glycine[a]	0	100	0
[^{13}C-2]glycine[b]	85	15	0
[^{15}N]glycine[a]	0	100	0
[^{13}C-1]sodium pyruvate[c]	0	100	0
[^{13}C-2]sodium pyruvate[c]	0	0	100
[^{13}C-3]sodium pyruvate[c]	0	0	100

[a] Glycine/pyruvic acid model system (3:1). [b] Glycine/pyruvic acid model system in the presence of unlabeled glyoxylic acid or paraformaldehyde. [c] Glycine·HCl/sodium pyruvate model system.

Tentative Identification of 3-Amino-4,5-dimethylfuran-2(5*H*)-one (1) and Intermediate 2. The sotolone precursor was identified by comparison of its retention time and mass spectrum to those generated from fenugreek seed and from commercial sotolone in the presence of excess ammonium chloride when pyrolyzed at 200 °C for 20 s and through NIST library matches in addition to isotope labeling data (see Tables 1 and 2). The data reported in Tables 1−3 are based on at least two replicate analyses with a percent standard deviation of <15%. The proposed structure of intermediate **2** was based on the expected label incorporation pattern and the mass spectral fragmentations shown in Table 3.

RESULTS AND DISCUSSION

In an effort to investigate the role of 2-keto acids such as glyoxylic and pyruvic acids in the Maillard reaction, glycine and

alanine were chosen as model amino acids and their reaction mixtures were analyzed using Py-GC-MS. The results of these investigations have indicated that such mixtures can generate 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) in addition to pyrazines and various other heterocyclic compounds that we reported previously.[16] Different model systems generated compound **1** in different intensities; however, the most efficient system was that of the glycine/pyruvic acid model (see Figure 2). The structure of compound **1** was confirmed through NIST library searches and by comparison of its retention time and mass spectrum to those generated from pyrolysis of fenugreek seeds and from the reaction of commercial sotolone with ammonium chloride. Both fenugreek seeds and the sotolone/NH_4Cl mixture generated peaks with retention times and mass spectra identical to those of the model systems and the NIST library with a very high match factor. As mentioned above, this compound was earlier reported[3] in the cultures of *L. helvus*, a mushroom from Europe producing sotolone, and in fenugreek seeds (*T. foenum-graecum*) by Peraza-Luna et al.[15] Due to the ease of conversion of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) into sotolone, a potent aroma compound, its formation mechanism was further investigated to identify the role of its important precursors. Figure 2 indicates the ability of various model systems studied to generate compound **1** and the origin of label incorporation from different precursors (see also Tables 1 and 2). According to Figure 2, unlike the glycine/glyoxylic model system, glycine/pyruvic acid and alanine/glyoxylic acid systems were capable of the formation of compound **1**. In addition, the glyoxylic acid/alanine model system seems to alter the mechanism of formation of **1** as indicted by the incorporation of four carbon atoms from the amino acid component as opposed to five carbon atoms from the keto-acid component in glycine pyruvic acid systems (see Figure 2 and Tables 1 and 2). These observations may indicate the presence of different pathways for the generation of **1**. To explore in detail the different pathways of formation of **1** and as indicated in Figure 2, variously labeled glycine, alanine, and pyruvic acid model systems were used. For model systems containing labeled pyruvic acid, glycine hydrochloride was used to neutralize the commercially available labeled sodium pyruvate. The efficiency of the pyrolytic generation of compound **1** was drastically reduced when the corresponding salts of glycine and pyruvic acid were used as indicated in Figure 2. Furthermore, complete label incorporation patterns from different model systems are reported in Tables 1 and 2.

Table 2. Percent Incorporation of Labeled Atoms from Alanine[a] in *m/z* 127 of 3-Amino-4,5-dimethyl-2(5*H*)-furanone (1)

labeled reactant	M	M + 1	M + 2
[^{13}C-1]alanine[a]	0	0	0
[^{13}C-2]alanine[b]	0	0	100
[^{13}C-3]alanine[c]	0	0	100
[^{15}N]alanine[a]	0	100	0

[a] Alanine/glyoxylic acid model system (3:1). [b] Also implies the incorporation of C-3 atom of alanine. [c] Based on the incorporation of C-2 of alanine.

Table 3. Percent Incorporation of Labelled Atoms from Pyruvic Acid[a] in *m/z* 126 of 4,5-Dimethylfuran-2,3-dione (2)[b]

labeled reactant	M	M + 1	M + 2
[^{13}C-1]sodium pyruvate	0	100	0
[^{13}C-2]sodium pyruvate	0	0	100
[^{13}C-3]sodium pyruvate	0	0	100

[a] Paraformaldehyde/sodium pyruvate model system. [b] Retention time, 15.55 min; *m/z* (% intensity) 127 (14.2), 126 (100), 98 (15.2), 97 (22.5), 84 (27.7), 83 (59.9), 69 (40.3), 55 (60.8), 41 (27.6), 39 (46.6).

Proposed Mechanism of Formation of 3-Amino-4,5-dimethyl-2(5*H*)-furanone (1). On the basis of the analysis of label incorporation patterns in different model systems (Tables 1 and 2), we propose a two-step formation pathway for the title compound (Figure 3). The first step involves the formation of 4,5-dimethylfuran-2,3-dione (**2**), and the second step involves its conversion into **1** through interaction with any amino acid. This proposition is based on the observations that model systems lacking amino acids generated only compound **2** such as paraformaldehyde and pyruvic acid (Table 3) and that model systems containing amino acids generated only compound **1**. On the other hand, paraformaldehyde/glycine/pyruvic acid model

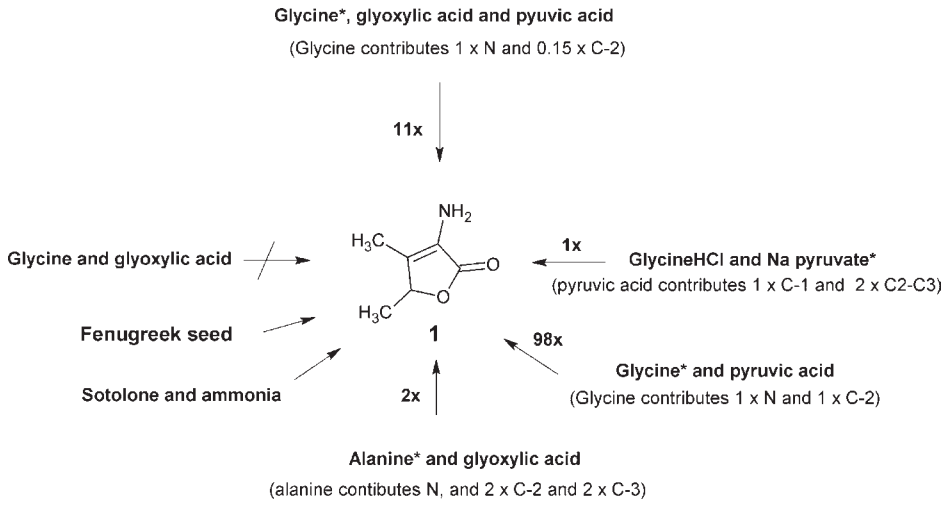

Figure 2. Different model systems capable of formation of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**). Asterisks indicate the labeled component in the reaction mixtures. Efficiencies of formation (indicated over the arrows) are based on area/mol of amino acid reactant relative to the least efficient glycine·HCl/sodium pyruvate model.

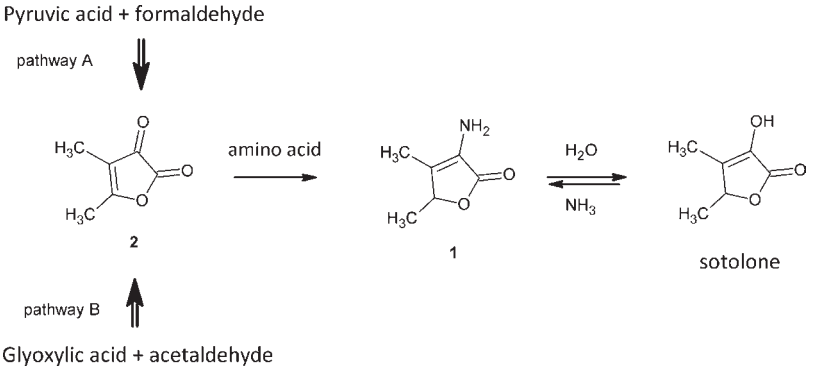

Figure 3. General pathways of formation of 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**).

Table 4. Comparsion[a] of Sotolone and 3-Amino-4,5-dimethyl-2(5*H*)-furanone (1) Formation in the Presence and Absence of Moisture

model system	sotolone	3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**)
paraformaldehyde/pyruvic acid/glycine (dry)	0	14.3×10^{10}
paraformaldehyde/pyruvic acid/glycine (wet)	19.3×10^{10}	0

[a] In area/mol of glycine (values are based on the average of duplicate analysis with % RSD < 15).

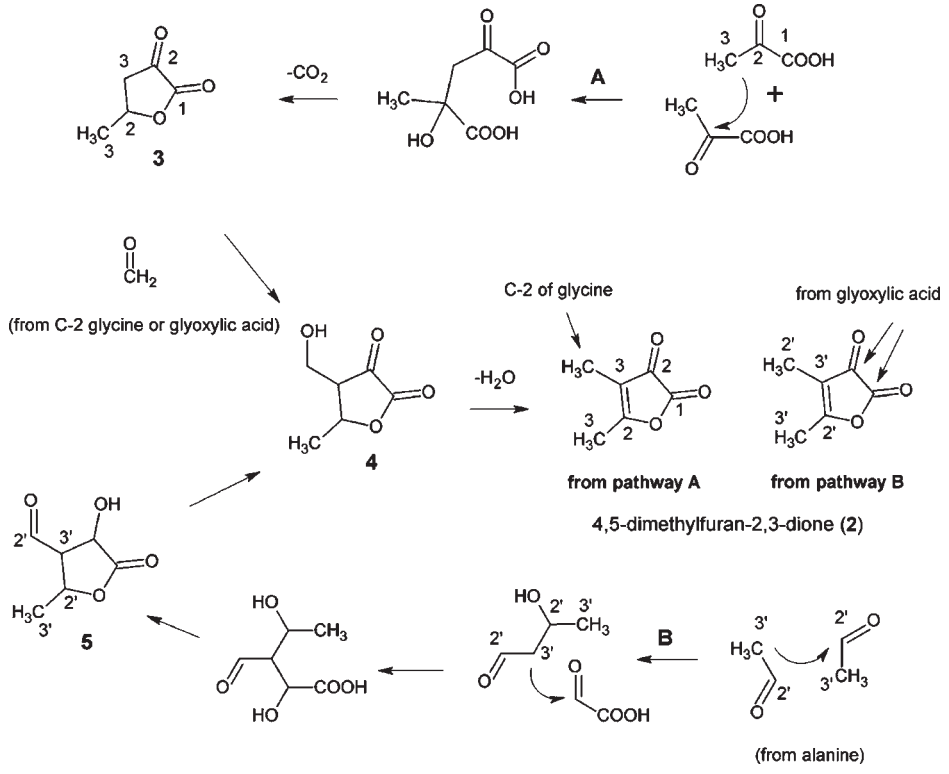

Figure 4. Proposed mechanism of formation of 4,5-dimethylfuran-2,3-dione through two pathways. Pathway A requires pyruvic acid and any formaldehyde source; pathway B requires glyoxylic acid and any acetaldehyde source.

systems when reacted in the presence of moisture generated only sotolone (see Table 4), confirming the ability of compound **1** to be hydrolyzed easily into sotolone. The data in Table 4 confirm the hypothesis that sotolone can be generated from 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) in the presence of moisture.

*Formation of 4,5-Dimethylfuran-2,3-dione (**2**).* On the basis of the label incorporation patterns of different precursors (Tables 1 and 2), the proposed intermediate 4,5-dimethylfuran-2,3-dione (**2**) can be formed by two different pathways shown in Figure 4. Pathway A requires only pyruvic acid and a formaldehyde source. In different model systems paraformaldehyde, glyoxylic acid, or glycine was used as a source of formaldehyde, because glycine in the presence of pyruvic acid is known to generate formaldehyde;[16] similarly, the decarboxylation of

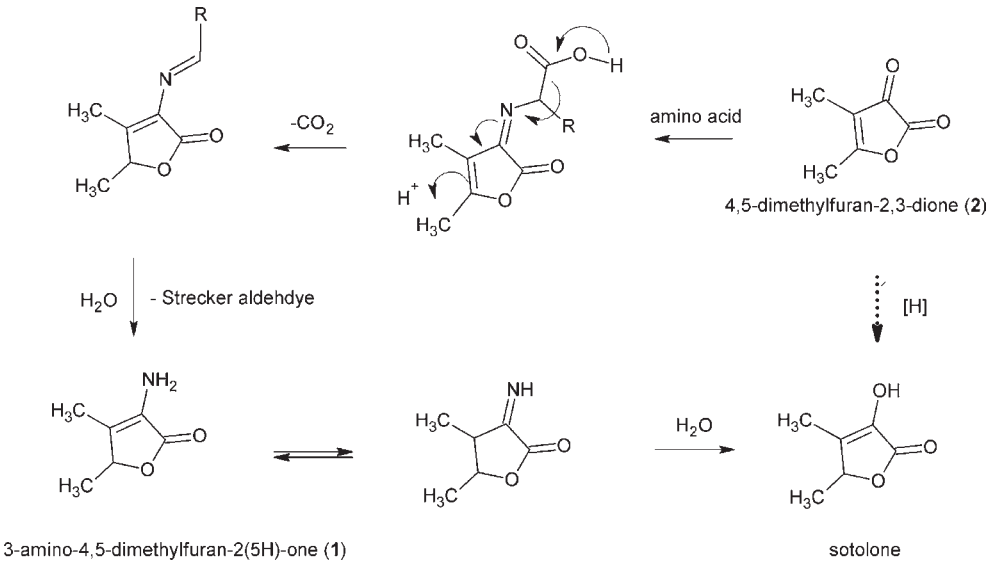

Figure 5. Proposed mechanism of conversion of 4,5-dimethylfuran-2,3-dione (**2**) into 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) through Strecker-like reaction. R, amino acid side chain.

glyoxylic acid can yield formaldehyde. In the absence of a nitrogen source, model systems containing paraformaldehyde and pyruvic acid, for example, generated only compound **2**, whereas in the presence of excess glycine, only compound **1** was detected, indicating conversion of **2** to **1** by the action of amino acid (see Figure 5). Pathway A requires aldol addition of two pyruvic acid molecules followed by decarboxylation and lactonization to form a furan-dione intermediate (**3**) that can undergo aldol addition with formaldehyde to form intermediate **4** followed by dehydration and isomerization to generate 4,5-dimethylfuran-2,3-dione (**2**). When selectively labeled pyruvic acids were reacted with paraformaldehyde as the source of formaldehyde, the compound (**2**) incorporated one C-1 and two C2−C3 atoms (see Table 3), consistent with the proposed pathway A in Figure 4. However, in the presence of [^{13}C-2]glycine as the source of formaldehyde, pyruvic acid generated compound **1** with the same incorporation pattern of carbon atoms of pyruvic acid in addition to 100% incorporation of C-2 atom of glycine. When unlabeled glyoxylic acid was also added as a source of formaldehyde, the extent of incorporation of the [^{13}C-2]glycine label was reduced to 15%, indicating the higher efficiency of glyoxylic acid to generate formaldehyde relative to glycine (Table 1). On the other hand, unlike model systems containing glyoxylic acid and glycine, the glyoxylic acid and alanine system also generated compound **1**, indicating the importance of alanine not only as a source of nitrogen but also as a precursor of the carbon atoms of the backbone of structure **1**. In pathway B (Figure 4) the self-aldol addition product of acetaldehyde undergoes a second aldol reaction with glyoxylic acid and the subsequent lactonization generates intermediate **5**, which can isomerize into **4** and, similarly to pathway A, dehydrates to generate compound **2**.

*Conversion of 4,5-Dimethylfuran-2,3-dione (**2**) into 3-Amino-4,5-dimethyl-2(5H)-furanone (**1**).* Both pathways A and B described above can generate compound **2**, which can undergo an interesting variant of the Strecker reaction in the presence of any amino acid where the α,β-unsaturated carbonyl moiety of **2** is converted into the eneamine moiety of **1**, releasing in the process the Strecker aldehyde (Figure 5). This transformation proceeds with retention of labeled carbon atom positions of intermediate **2** in the structure **1**. Structure **1** was confirmed not only through the NIST library search and mass spectral fragmentation patterns but also through the retention time of its naturally occurring counterpart desorbed from fenugreek seeds through pyrolysis and through its direct generation from the reaction of commercial sotolone with ammonia. Figure 4 summarizes the origin of the expected label incorporation in compound **2** from pathways A and B. Isotope label incorporation data supporting the proposed pathway A are shown in Table 1 and for pathway B in Table 2. Data in Table 1 indicate that in the presence of glycine and glyoxylic acid the main source of formaldehyde comes from glyoxylic acid decarboxylation (85%) and that there is 100% incorporation of one nitrogen atom from glycine. In addition, the data in Table 1 also confirm that the remaining five carbon atoms originate from pyruvic acid and as predicted from the proposed pathway, only one C-1 atom and two C2−C3 fragments were incorporated into the structure **1** as shown in Figure 4. With regard to pathway B, data in Table 2 indicate incorporation of a single nitrogen atom of alanine and no incorporation of C-1 atom, but double incorporation of C-2 and C-3 atoms from alanine, consistent with the proposed pathway B shown in Figure 4. Of six carbon atoms of structure **1** four were supplied by alanine, indicating the remaining two atoms originated from glyoxylic acid as proposed by pathway B shown in Figure 4. This pathway, although minor, may also shed some light on the mechanism of formation of sotolone as an off-flavor in citrus-based soft drinks containing ascorbic acid during storage.[17] In this study ethanol, along with ascorbic acid, was identified as a precursor of sotolone through incorporation of either 1 or 2 mol of ethanol into the sotolone backbone. Ascorbic acid is known to generate glyoxylic acid when incubated under storage conditions,[18] and oxidation of ethanol into acetaldehyde during storage of alcoholic beverages has been documented,[19] indicating the possibility that pathway B shown in Figure 4 can provide a rationale for the incorporation of 2 mol of ethanol into the sotolone backbone in citrus-based soft drinks containing ascorbic acid.

Condensation of different keto-acids, dicarbonyl compounds, and amino acids has been proposed in the literature as precursors

of sotolonel; however, identification of a nitrogen-containing precursor such as 3-amino-4,5-dimethyl-2(5*H*)-furanone (**1**) that can be thermally generated from pyruvic acid and glycine has not been reported so far. This knowledge may be used to allow controlled generation of the intense aroma of sotolone in selected food products as demonstrated by the ability of pyruvic acid/glycine model system to generate sotolone when reacted in the presence of moisture.

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